Supporting Online Material for

Hybrid Neurons in a MicroRNA Mutant Are Putative Evolutionary Intermediates in Insect CO_2 Sensory Systems

Pelin Cayirlioglu, Ilona Grunwald Kadow, Xiaoli Zhan, Katsutomo Okamura, Greg S. B. Suh, Dorian Gunning, Eric C. Lai, S. Lawrence Zipursky*

*To whom correspondence should be addressed. E-mail: lzipursky@mednet.ucla.edu

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Materials and methods

Genetics and Molecular Biology

Fly stocks were maintained in standard medium. P-element mutants were obtained from Bloomington or Szeged stock centers and were recombined to FRT chromosomes by the UCLA undergrad consortium (*1*).

The genetic screen was done using FRT/FLP mosaic analysis and MARCM analysis as described previously (*2*). To generate large antennal and maxillary palp (MP) clones eyeless-FLP was used in combination with a cell lethal mutation on 3R. Mutations (P-element) on the right arm of chromosome 3 were screened using the following genotype: *w-; Or-syt-GFP/+; FRT82 mutation/FRT82 CL GMR-hid ey-GAL4 UAS-FLP*. MARCM analysis was carried out on flies of the following genotype: *eyFLP; Or-gal4 UAS-sytGPF (or UAS-mCD8GFP)/+; FRT82 mutation/FRT82Gal80E2F*. ORN labeling was achieved by fusing the promoter-elements to GAL4 or directly to synaptotagmin-GFP (*3*). Promoters or fly stocks were generously provided by the labs of Leslie Vosshall (Rockefeller University) and Barry Dickson (IMP, Vienna). P-element plasmid rescue was performed as described previously (*4*). MPS-GAL4 driver is a promoter fusion of Or59c 5' with GAL4. This was the original Or59c driver (*3*). However, our

characterization of the wiring pattern as well as in situ experiments examining the odorant receptor expression of the ORNs that express this driver (*5*) suggest that this is an unfaithful driver that is expressed in additional ORNs in the MP. This driver was used in cell count and pupal developmental studies in the maxillary palps. The fibers labeled with this driver innervate two major glomeruli targeted by Or59c and Or42a ORNs. Occasionally, in some of the brains, a third glomerulus that corresponds to Or85d cognate glomerulus also is slightly labeled. Our developmental study suggests that it is expressed in single cells within a pb1 and pb3 sensilla in wild type MP that harbor Or42a and Or59c ORNs, respectively.

As the *S0962-07* mutation is organismal lethal, all analyses were done in mosaic animals where more than 50% of cells in the antenna and the MPs were homozygous mutant while the rest of the animal was wild. Gr21a transcriptional reporter was used for CO_2 neurons (i.e. Gr21a-GAL4 driving membrane-bound GFP (UAS-mCD8GFP)), to detect cell bodies in the antenna and MP.

miR-279 deletions were generated using P-element excision of two different P-element insertions. We used standard methods to generate ~200 excisions each from *EP(3)3626* and *EP(3)3069*, which are both inserted downstream of *miR-279*. This yielded a 1.9kb deletion from *EP(3)3069* and 1.2kb and 0.8kb deletion alleles from *EP(3)3626*. Genomic sequencing revealed that the *miR-279* locus is cleanly removed in the two larger deletion alleles. *UAS-nerfin* transgenic flies and *nerfin-1* loss of function alleles were generously provided by Ward Odenwald. miR-279-GAL4 and *miR-279* genomic transgenes were generated using these primer sequences: MIR279FOR1: 5’

TAT TTT TGC GCC TGC CAA TAA GCG 3' MIR279REV1: 5' TAG ATC AGT GAC TCA GCT GGC AAC 3' MIR279FOR2: 5' GTA TTC AAC GCG TGT TTT CTG 3' MIR279REV2: 5' AAT CGG AAT CGG AAT CAG AAT CGC 3' MIR279FOR3: 5' TGA AAA TAC GCG TAT GGA AAT GCC 3' MIR279REV3: 5' CAG CTC CAG TCC CAA TTC C 3'

miR-279 mutant phenotype is 100% penetrant. Genomic rescue experiments were carried out such that *eyflp; +/[miR-279];FRT82 S0962-07/ TM6B* males were crossed to *Gr21-Gal4,UAS-mCD8GFP/CyO; FRT82Gal80E2F/TM2* virgin females. Only half of the progeny that was dissected was expected to have the rescue transgene. Our quantifications show that more than half of the dissected progeny show no phenotype suggesting complete rescue of the mutant phenotype. The rescue observed for the wiring phenotype in the brain, and the generation of ectopic Gr21a cells in the MP were, 60% (n=25) and 67% (n=30) of all analyzed progeny, respectively.

miR-279-GAL4 transcriptional reporter comprised the entire 5' upstream sequence of *miR-279* from the genomic rescue fragment fused to the transcriptional activator GAL4.

Genotypes for mutant analysis:

Control:

eyflp;Gr21-Gal4,UAS-mCD8GFP/+;FRT82/FRT82Gal80E2F.

eyflp;Gr21-sytGFP/+;FRT82/FRT82Gal80E2F

eyflp;MPS-GAL4UAS-mCD8GFP/+;FRT82/FRT82Gal80E2F

eyflp;Or-Gal4,UAS-mCD8GFP/+; FRT82/FRT82Gal80E2F

Mutant:

eyflp; Gr21-Gal4,UAS-mCD8GFP/+;FRT82S0962-07/FRT82Gal80E2F

eyflp;Gr21- sytGFP/+;FRT82S0962-07/FRT82Gal80E2F

eyflp;MPS-GAL4UAS-mCD8GFP/+;FRT82S0962-07/FRT82Gal80E2F

eyflp; Or-Gal4,UAS-mCD8GFP/+;FRT82S0962-07/FRT82Gal80E2F

eyflp; Gr21-Gal4,UAS-sytGFP/+; FRT82 miR-279 $^{\Delta 0.8}$*/FRT82Gal80E2F*

miR-279 rescue:

eyflp; Gr21-Gal4,UAS-sytGFP/[miR-279];FRT82 S0962-07/FRT82Gal80E2F

Nerfin-1 genetic interactions:

eyFLP;Gr21a-GAL4,UAS-mCD8GFP/+;Df(2)nerfin-1^{154}*FRT82S0962-07/FRT82GAL80E2F* (nerfin^{154} *miR-279*^{P}).

eyFLP;Gr21a-GAL4,UAS-mCD8GFP/+;FRT82S0962-07/FRT82GAL80E2F (*miR-279*^{P}).

Electrophysiology:

Mutant:

eyflp; Gr21-Gal4,UAS-mCD8GFP/+;FRT82S0962-07/FRT82Gal80E2F

Control:

Canton S.

Electrophysiology

Flies were mounted on a glass slide and viewed with an Olympus BX51WI microscope at 1000X magnification. Electrical signals were recorded with a Multiclamp 700B amplifier and Digi-data 1322A at the sampling rate of 10kHz, stored in a PC with pClamp 9.2 software, and plotted with Clampfit 9.2 and SigmaPlot 9 software as described(*6*). Recordings were performed by placement of a glass electrode onto the patch of GFP+ cells on the lateral side of the palp with a reference electrode inserted in the eye.

Ectopic neurons showed a weak but significant response to CO_2. By contrast, wild type MPs showed no respone (see Fig.1). These data suggest that there are differences between these neurons and wild type CO_2 neurons in the antenna. These differences in the hybrid neurons could include lower levels of receptor expression, inhibitory effects of MP olfactory receptors that are co-expressed, or the lack of certain co-factors downstream or upstream Gr21a/Gr63a.

Immunohistology

Staining was performed as previously described (*3*). Primary antibodies were used in the following dilutions: Guinea pig anti-Nerfin-1 antibody (provided by the Odenwald lab), 1:1000; mouse anti-NC82 (DSHB), 1:20; mouse anti-prospero (DSHB),

1:20; mouse and rat anti-elav (DSHB), 1:100; and rabbit and mouse anti-GFP, 1:2000 and 1:500, respectively. All secondary antibodies were used at 1:200.

Whole mount in situ hybridization

Adult probiscises were fixed in 4% Paraformaldehyde, 0.05% Tween-20 for 30 min at room temperature. Prehybridization and hybridization were done at 55˚C in 50% Formamide, 5xSSC (pH 4.5), 0.1mg/ml yeast tRNA, 0.05 mg/ml Heparin, 0.1% Tween20 for 1 hr and overnight, respectively. Probes were generated using digoxigenin-RNA labeling kit. Probe templates were kindly provided by Leslie Vosshall. After hybridization proboscises were washed 5 times in hybridization buffer at 55˚C, followed by 3 times 5 min washes in 0.1M Tris pH7.5, 0.15M NaCl, and 0.05% Tween-20. Samples were blocked in 0.1M Tris pH7.5, 0.15M NaCl, 0.05% Tween-20, and 0.5% blocking reagent TSA Biotin kit and incubated overnight in anti-dig Peroxidase (1:200) and anti-GFP-alexa488 antibodies (1:200). To amplify the RNA probe signal, samples were incubated in biotinyl tyramide (1:100) for 2 hrs at room temperature, followed by incubation in streptavidin-cy3. After subsequent washes in 0.1M Tris pH7.5, 0.15M NaCl, and 0.05% Tween-20, samples were mounted with Vectastain and analyzed using a confocal microscope.

PicTar sequence comparison

All the computational analysis and sequence comparison was done using the PicTar program that can be accessed via: http://genome.ucsc.edu/cgi-bin/hgTrackUi?db=hg17&g=picTar or http://pictar.bio.nyu.edu/.

Luciferase assay

To generate luciferase targets, we amplified a 1.8kb *nerfin* fragment (including the entire 3' UTR and 220bp of downstream sequence), and a 550bp *nerfin* 3'UTR fragment starting at position 680 of the 3' UTR ("nerfin 680", including three miR-279 sites) and cloned these downstream of the renilla luciferase coding region in psiCHECK2; this vector contains an internal firefly luciferase gene that serves as an internal control. The inclusion of downstream genomic sequences in the full-length constructs permits the evaluation of miRNA-mediated regulation of endogenously-terminated transcripts. For the *miR-279* expression construct, we cloned 415 bp of genomic sequence, centered on the *miR-279* hairpin, into the 3' UTR of UAS-DsRed (*7*). Different 3'UTRs were fused to a luciferase reporter construct. psiCheck, a control 3'UTR; nerfin, the entire *nerfin-1* 3'UTR containing four 8mer (blue boxes) and two 7mer (light blue boxes) *miR-279* binding sites (*8*); nerfin680, a shorter piece containing the major cluster of three 8mer miR-279 sites. Primer sequences are available upon request. We then transfected 25 ng target, 12.5 ng ub-Gal4 and 25 ng UAS-DsRed-miR-279 plasmids into 1x10[5] S2 cells in 96 well format. Three days later, we lysed the cells and subjected them to dual luciferase assay and analyzed these on a plate luminometer. Quadruplicate transfections were performed on two different batches of cells on different days, and the data were pooled in the figure shown.

Luciferase sensor assays were performed as previously described (*9*). We performed quadruplicate transfections of 25 ng target, 12.5 ng *ub-Gal4* and 25 ng *UAS-DsRed-miRNA* plasmids into $1x10^5$ S2 cells in 96 well format. For 2'Omethyl antisense

mediated de-silencing assay, we introduced 25ng target plasmid and 5 pmol (50nM) or 10pmol (100nM) of 2'Ome oligonucleotides for each well (miR-279ASO: GTTATTAATGAGTGTGGATCTAGTCACAAAT, miR-124ASO: GCTCTTGGCATTCACCGCGTGCCTTATGGTG). Three days after transfection, we lysed the cells and subjected them to dual luciferase assay and analyzed these on a Veritas plate luminometer.

Fig. S1: Characterization of ectopic neurons in *S0962-07* mutants.

(A) Average number of Elav-positive cells in wild type and mutant pupal MPs. ** $p < 0.001$. MPs scored: wild type, n = 7; mutant, n = 8. **(B)** Mutant and control MPs labeled with Or83b-GAL4, UAS-mCD8GFP. Single neurons or clusters of up to 2 cells were observed in the control, while also clusters of up to 4 cells were found in the mutant palps. **(C)** Quantification of Or83b-positive cells in mutant and control palps. Approximately 10 clusters containing 3-4 positive cells were observed in the mutant compared to controls (n = 11 (control), n = 14 (mutant), ** $p < 0.0001$). The number of 1-2 cell clusters was reduced in the mutant compared to control, while the number of total cell clusters was unchanged compared to control palps. This suggests that additional neurons are added to existing sensilla. (B,C) *Genotypes: eyflp; Or83b-Gal4,UAS-mCD8GFP/+; FRT82 / FRT82Gal80E2F* (control). *eyflp; Or83b-Gal4,UAS-mCD8GFP/+;FRT82 S0962-07/ FRT82Gal80E2F* (*miR-279*^{P})

Fig.S2: The adult Drosophila olfactory system.

Neurons expressing the CO_2 receptors, Gr21a/Gr63a (orange) reside in the antenna (an) and project to the V-glomerulus in the antennal lobe (al). Or59c and Or42a expressing neurons (green) in the MP innervate two medial glomeruli.

Fig.S3: *S0962-07* gene encodes a microRNA.

(A) A P-element (green triangle) insertion maps 1kb upstream of *miR-279* in *S0962-07*. Deletion mutants, red lines; 3kb genomic region used in the rescue experiments and miR-279-GAL4 reporter construct, blue lines. **(B, C)** Gr21a targeting to the medial antennal lobe. **(B)** and ectopic Gr21a cells in the MP **(C)** are rescued by the *miR-279* rescue transgene. Both antennae and MP are present in these studies. Genotypes: (B) (from top to bottom) *eyflp; Gr21-Gal4,UAS-sytGFP/+; FRT82 / FRT82Gal80E2F* (control). *eyflp; Gr21-Gal4,UAS-sytGFP/+;FRT82 S0962-07/ FRT82Gal80E2F* (*miR-279^{P}*). *eyflp; Gr21-Gal4,UAS-sytGFP/+; FRT82 miR-279 $^{\Delta 0.8}$ / FRT82Gal80E2F* (miR-279$^{\Delta 0.8}$). *eyflp; Gr21-Gal4,UAS-sytGFP/[miR-279];FRT82 S0962-07/ FRT82Gal80E2F* (*miR-279* rescue). (C) *eyflp; Gr21-Gal4,UAS-mCD8GFP/+;FRT82 S0962-07/ FRT82Gal80E2F* (*miR-279^{P}*). *eyflp; Gr21-Gal4,UAS-mCD8GFP/[miR-279];FRT82 S0962-07/ FRT82Gal80E2F* (*miR-279* rescue).

Fig.S4: Elav, 22c10 and Prospero expression in *miR-279* positive cells.

miR-279 is expressed in big cells that appear to be similar to sensory organ precursors (SOP), which give rise to the components of individual sensilla through rounds of cell divisions. However, these precursors appear different from previously described antennal SOPs (*10*).

A) miR-279-GAL4, UAS-mCD8GFP (green) expressing MPs co-stained with anti-Elav antibody (red) at 40-50 hrs and 50-60 hrs APF. Lower panel is a higher magnification of a cell cluster at 50-60 hrs APF. Similar to the observations in the antennal precursors (*10*), Elav protein is detected in the precursors before neuronal differentiation. Arrows denote unequal distribution of Elav within miR-279 expressing clusters. Arrowheads point to ORNs that have undergone neuronal differentiation, and no longer express miR-279. **(B)** miR-279-GAL4, UAS-mCD8GFP (green) and 22C10 (red) on pupal MPs (50-60 hrs APF). 22C10 labels Futsch protein, which is expressed in postmitotic neurons. **(C)** anti-Prospero staining (red) on miR-279-GAL4, UAS-mCD8GFP (green) pupal MPs (50-60 hrs APF). All these neuronal markers are initially expressed in the SOP. Later, they become unequally distributed as multiple cell clusters form through presumed cell divisions from the original SOP. The identity of these cells as they divide is unknown, due to lack of information on the development of MPs, and of developmental markers that label different cell types within the cluster.

Fig.S5: Nerfin-1 and miR-279 expression in developing MP.

Pie charts showing quantification of **(A)** miR-279-GAL4,UAS-mCD8GFP and Nerfin-1 protein distribution in developing MP and **(B)** of Nerfin-1 protein expression in ectopic Gr21a cells in mutant MPs. (** $p < 0.001$).

Fig.S6: Suppression of Nerfin-1 expression requires specific *miR-279* target sequences.

(A) *miR-279* binding sites within *nerfin-1* 3'UTR are conserved in different *Drosophilids*. **(B)** Antisense oligos directed against the core recognition sequence of *miR-279* or *miR-124* were co-transfected with the nerfin-1 3'UTR luciferase reporter into S2 cells. *miR-279* specific antisense oligos relieved the repression on Nerfin-1 expression monitored with the 3'UTR nerfin-1 luciferase reporter. *miR-124* antisense oligos had no effect on nerfin-1 reporter repression. This also shows that *miR-279* specifically counteracts endogenous *miR-279* that is expressed in S2 cells, and further strongly suggests that the predicted *miR-279* core sequences in the 3'UTR are required for suppression by *miR-279.* We also note that Odenwald and colleagues recently reported complementary evidence for direct repression of *nerfin-1* by *miR-279* in the Drosophila embryo (*11*).

Fig.S7: Effect of Nerfin-1 on the number of Gr21a cells.

(A) The number of antennal Gr21a-positive ORNs is not affected by removal of one copy of *nerfin-1*. Gr21a expression is not seen in wild type palps, or wild type palp heterozygous for the Nerfin^{154} allele. *Genotypes: Gr21a-Gal4,UAS-mCD8GFP/+;TM2/TM6B* (wild type), *Gr21a-Gal4,UAS-mCD8GFP/+;nerfin^{154}/TM6B* (Nerfin^{154}/+). **(B)** Overexpression of Nerfin-1 in ey-flp clones using the miR-279-GAL4 driver construct did not result in a miR-279-like phenotype. *Genotype: eyflp; UAS-nerfin-1/miR-279-GAL4;FRT82Gr21a-sytGFP/FRT82Gal80E2F.* **(C)** Miswiring of Gr21a neurons still persists in flies where the dose of Nerfin-1 in miR-279 mutant background is reduced to half. These fibers presumably originate from the cell bodies that are still present in the MP (see Figure 3G). Genotype: *eyflp; Gr21a-GAL4,UAS-mCD8GFP/+; nerfin^{154}FRT82 miR-279^{P}/FRT82Gal80E2F.*

References

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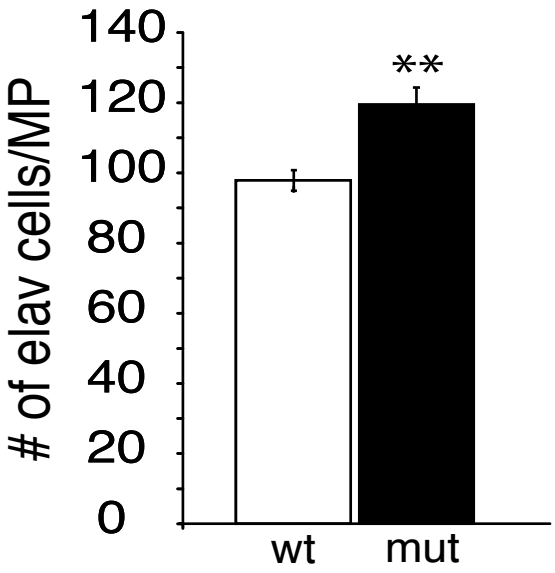


B

C

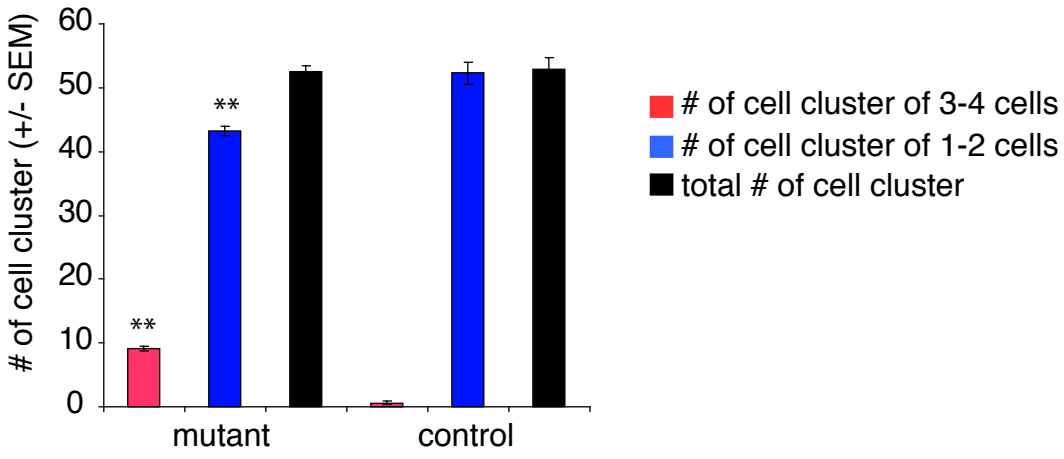
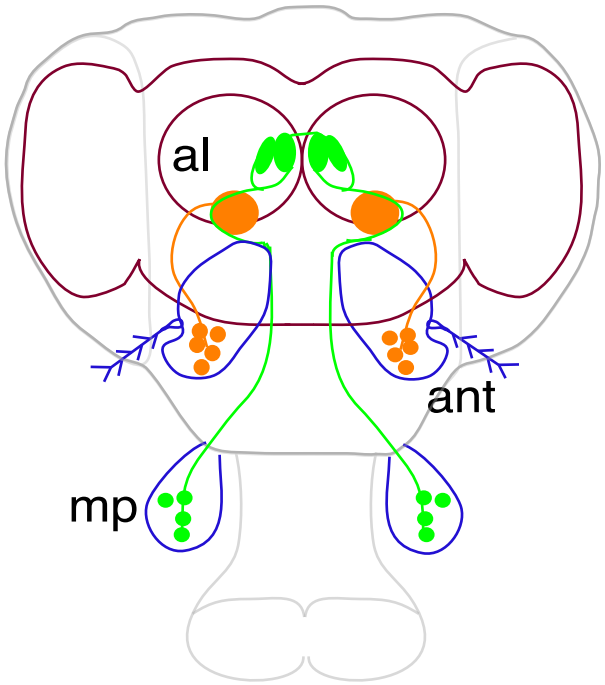


Figure S1



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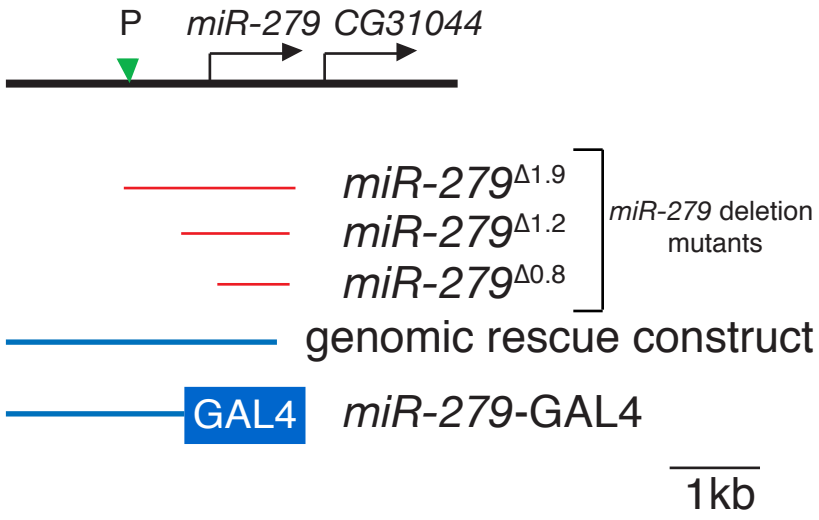

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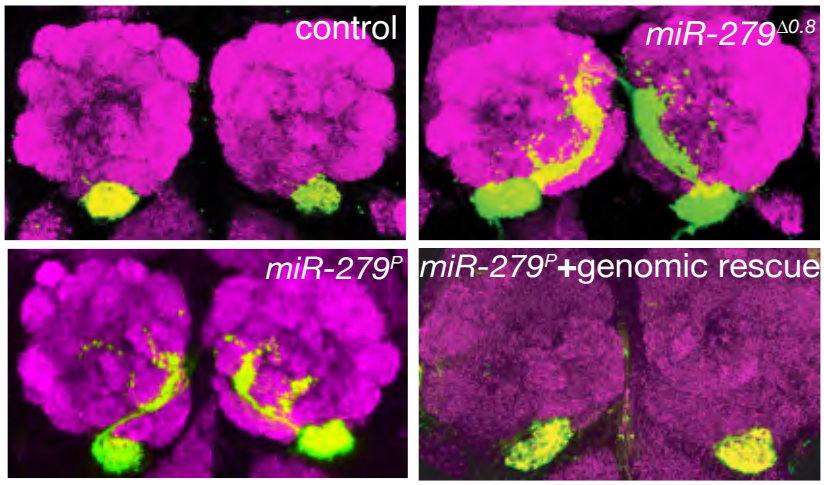

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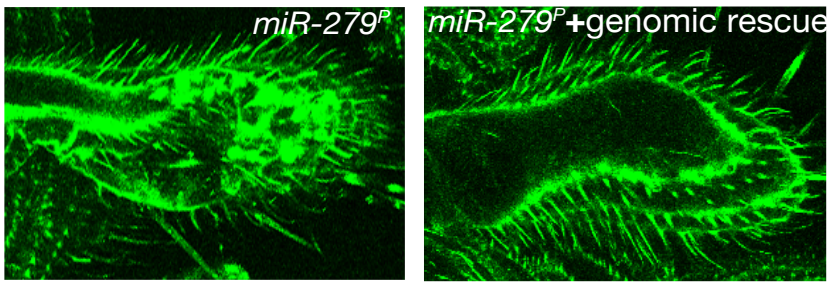

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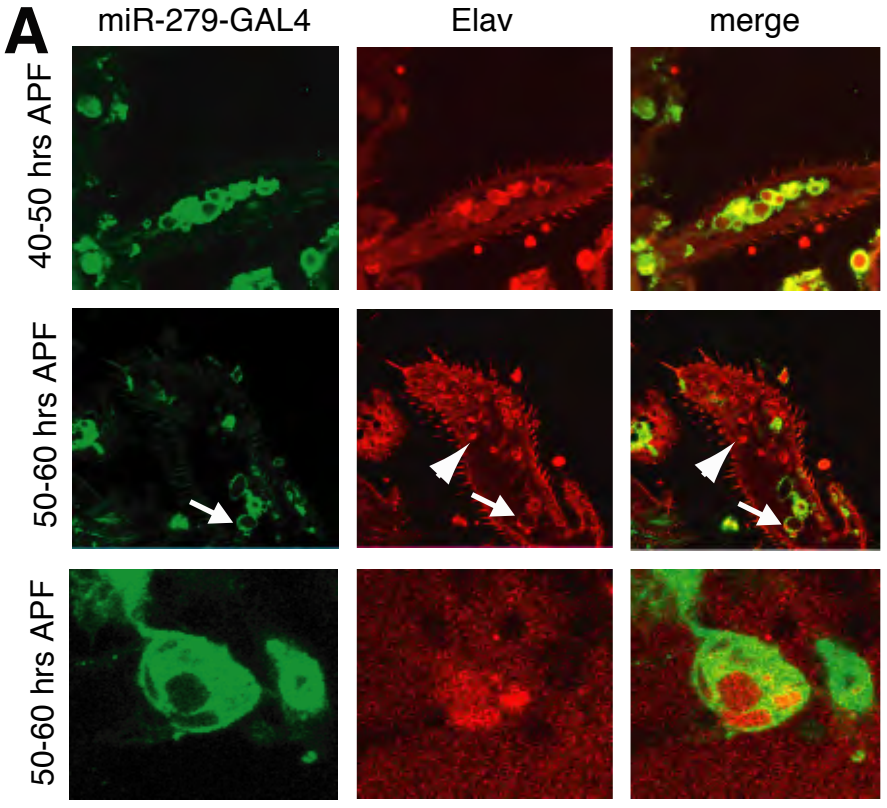
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miR-279-GAL4
Elav
merge
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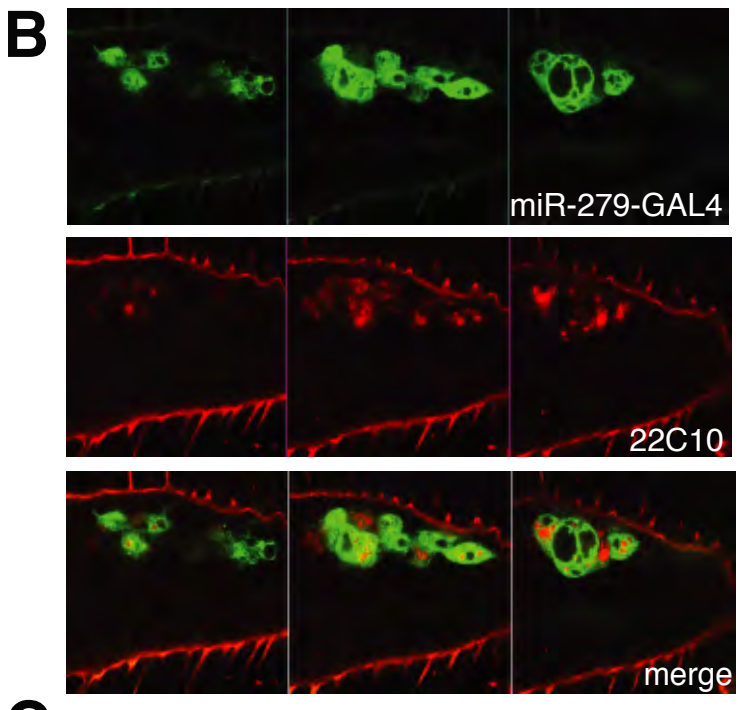
50-60 hrs APF
50-60 hrs APF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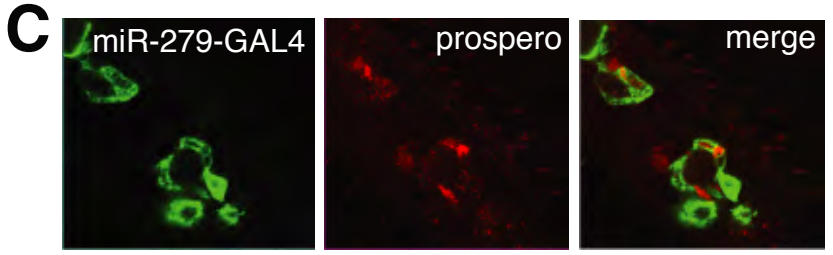
22C10
merge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miR-279-GAL4
prospero
merge

Figure S4.

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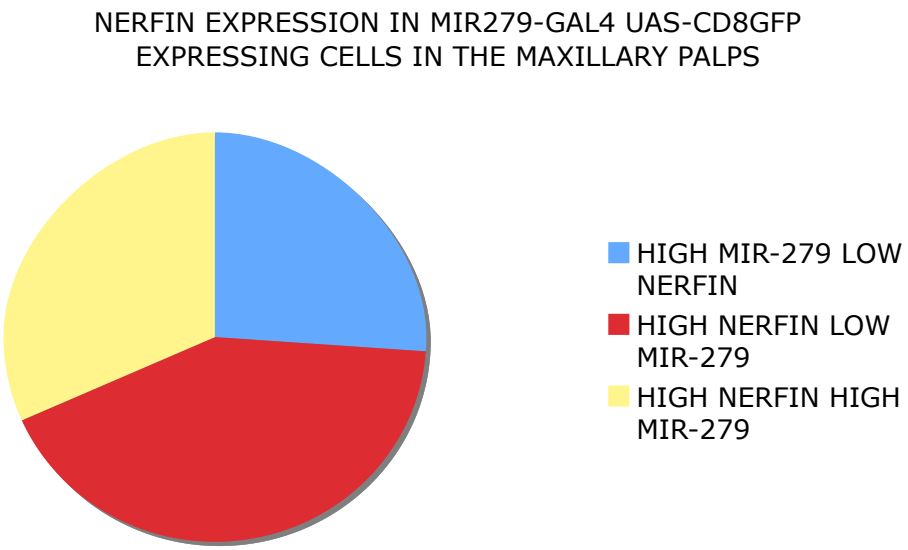

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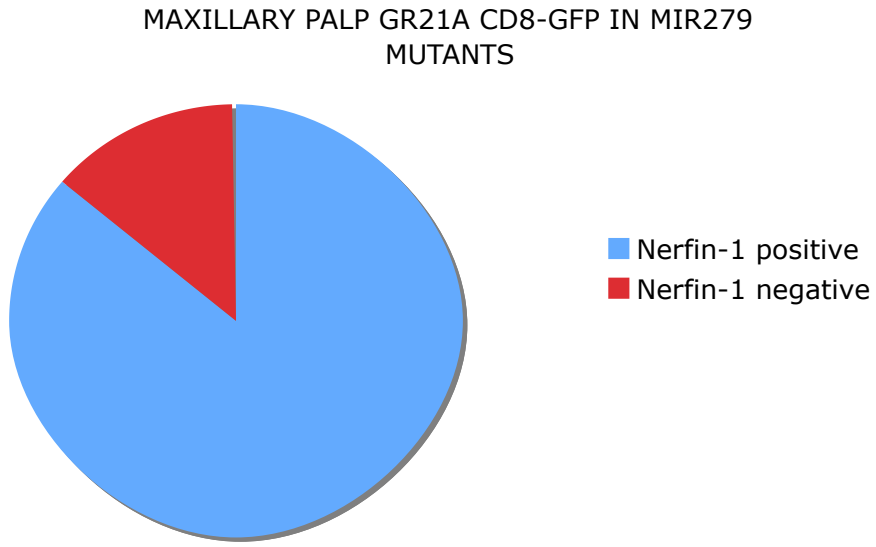

$p<0.005$

Figure S5.

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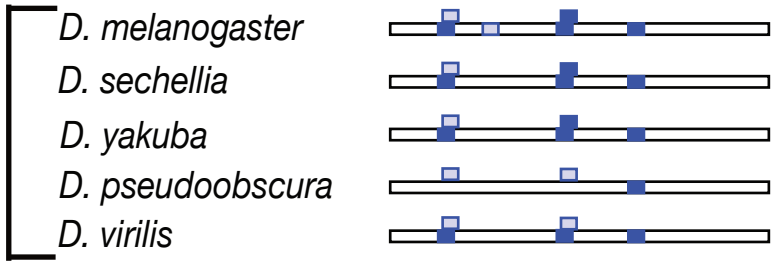

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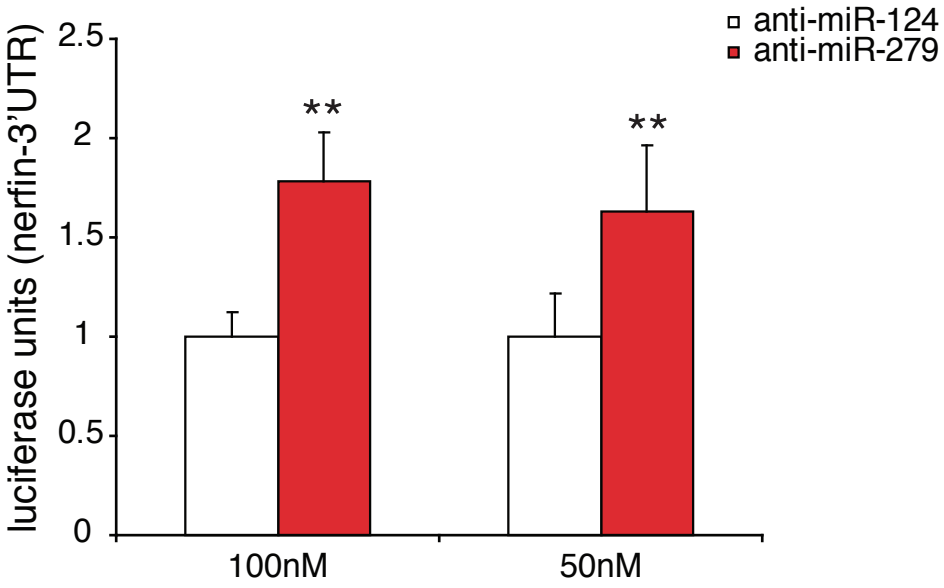
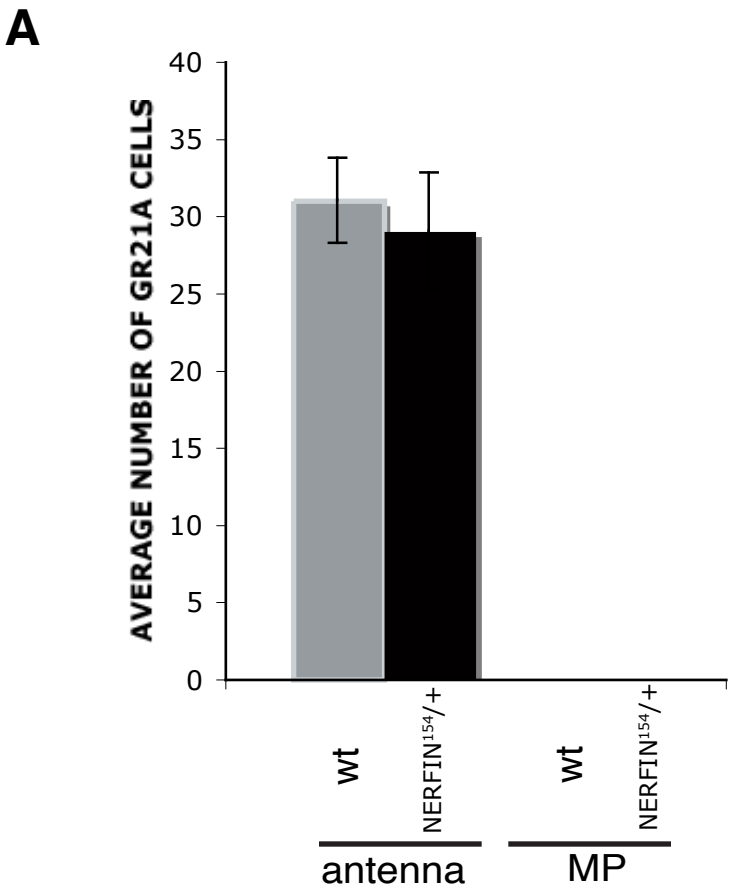


** p<0.001

Figure S6.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AVERAGE NUMBER OF GR21A CELLS
40
35
30
25
20
15
10
5
0
wt
NERFIN154/+
wt
NERFIN154/+
antenna
MP
B
miR-279::nerfin-1
C
nerfin154 miR-379P
+ miR-379P

Figure S7.